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I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 9882 for a patent by ADELAIDE UNIVERSITY as filed on 09 January 2002.

I further certify that the name of the applicant has been amended to THE UNIVERSITY OF ADELAIDE pursuant to the provisions of Section 104 of the Patents Act 1990.

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Twenty-fourth day of January 2003

JONNE YABSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

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PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED

Invention title: ANIMAL HUSBANDRY HAIR REMOVAL METHOD

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The invention is described in the following statement:

This invention relates to a method for the removal of hair for animal husbandry purposes.

The removal of hair from certain regions of the body of some animals is considered an important animal husbandry activity. Sheep raised for wool, such as merinos, have been bred to enhance wool production, and thus the more wool borne by the sheep the greater the economic return. Part of the breeding process has involved enhancing loose skin characteristics to increase the number of hair follicles and thus yield of wool. A side effect of not only the loose skin but also the greater production of wool is that the breech of sheep where it is not appropriately maintained is readily subjected to urine staining, faecal soiling or dags. Excessive moisture in the skin folds also results in bacterial growth and an odour that is an attractant for the gravid blowfly female to lay eggs, resulting in an enhanced fly strike rate. Breech strike, as it is known is the primary form of blowfly strike accounting for more than 80% of all blowfly strikes.

While crutching of sheep at appropriate times of the year reduces the incidence of breech strike, a significant number of sheep still become struck in this region. An operation pioneered by J H W Mules was introduced in the 1930's to remove folds of skin in the breech and to reduce the amount of wool on the breech, hence the amount of faecal and urinary soiling of the region. The Mules operation has been widely adopted by Merino sheep producers. Approximately 20 million lambs are mulesed nationally each year.

The original operation involved pinching a fold of skin on either side of the perineal area with Burdizzo pincers and cutting the fold off with a knife. This operation was considered, at the time, not to be painful because of the pressure of the pincers. Later the Mules operation was extended to remove skin from the tail, this was referred to as the 'Modified Mules operation.' The pincers and knife were replaced with blade shears to perform the operation. In Western Australia the mulesing contracting industry extended the area of skin removal in the crutch area as well as performing a total strip of the tail skin - the so called "Radical Mules Operation."

Apart from welfare concerns the Radical Mules Operation results in secondary problems such as a large wound area, increasing the chances of infection, secondary joint infections, wound contraction and distortion of tail and vulva. The longer-term problem of an increase in UV light induced skin cancer of the perineal region also became evident.

An alternative to the Mules operation is considered a high priority by the Merino sheep industry due to mounting consumer and animal activist pressure to improve animal welfare, but at present no such alternative exists.

SUMMARY OF THE INVENTION

The present invention has resulted in a simple approach to the long term removal of hair for animal husbandry purposes. The method involves introducing a collagen cleaving agent beneath the surface of the skin of a live animal in a form that provides a depilation effect.

In particular this invention differs from all depilatory methods for use on live animals known to the inventor in that this is the only method known to utilise the depilatory and perhaps also hair growth inhibitory properties of a collagen cleaving agent.

Experimentally it has been found that by an injection of 0.1ml of an aqueous solution of collagenase at a concentration in the order of 0.01% (w/v) or greater when introduced into the dermis in the crutch of a sheep has the effect of depilating an area estimated to be about $5cm^2$. The collagenase spreads through the dermis from the point of injection, and is believed to disrupt the collagen network which is thought to be essential for hair follicle attachment in the skin, and also for normal hair growth processes. A serous scab forms at the depilation site and on falling off, the hair and hair follicles are taken with the serous scab leaving a depilated area. In trials to date the depilation has been effective for a period of about 4 months with no sign of regrowth as yet. It is anticipated that such a depilation procedure might not need to be repeated throughout the life of the sheep, or other animal to be treated, however it might be necessary to repeat the procedure periodically.

The efficacy with which the exemplified procedure has worked, with minimal damage to surrounding tissue together with the long term nature of the hair removal effect is unexpected.

It is thought that the action of collagenase or other matrix metalloproteinases on hair growth is two-fold: the first is that a fine collagen network anchors the follicle within the dermis so disruption of this anchoring network results in liberation of the entire follicle from the skin. The second is that collagen matrix is involved in the normal functioning of the dermal papilla cells located in the base of the hair follicle. Disruption of the matrix will alter normal fibre-producing processes in the follicle bulb, so that fibre growth is inhibited, possibly permanently.

Experiments to date have shown that a number of the family of matrix metalloproteinases are capable of cleaving collagen and therefore having the effect. These include MMP-3 (also known as stromelysin), but the preferred collagen-cleaving agent is crude collagenase, comprising a mixture of collagenases. It will be understood that there are a large number of collagenases available and that the invention is not restricted to any one particular collagenase and therefore it is contemplated that most if not all of the available collagenases can be used in the practice of the invention. The primary limitation on the selection of collagenase is that it is able to weaken the adhesion that the connective tissue provides between the follicle and the surrounding dermis within a time period in which damage to the surrounding dermis is minimised. In a particularly preferred form of the invention the collagen cleaving enzyme is a mixture of at least two collagenases selected from the list of bacterial collagenases including Type IV, Type II, Type XI, Type I, Type VIII and Type V. These enzymes may be from *Clostridium histolyticum* and may be available commercially. The mixture of collagenases may also contain other proteases. It will be understood that the collagenases of matrix metalloproteinases may be altered proteins such as trunction, mutant or deletions.

In the case of collagenases or other matrix metalloproteinases, the enzyme(s) may also be used in conjunction with a source of divalent cations such as Zn^{2+} or Ca^{2+}.

The collagen cleaving enzyme may a protease, or a truncation, mutant or deletion thereof. However, there are a large number of available proteases and it is possible that there may be some proteases that are able to weaken the adhesion the connective tissue provides between the follicle and the surrounding dermis within a time period in which damage to the surrounding dermis and to the hair fibre or follicle is minimised.

Experiments to date suggest that levels of enzyme(s) in the composition, at least for the crude collagenase preparation, may need to be greater than about 0.01% w/w. It is known that 0.001% appears not to be effective however with suitable delivery methods or using enzymes with higher specific activities such levels may still be effective. Empirical trials will readily determine an appropriate level of enzyme to be added for effective removal of hair. Similarly with such trials it will be readily determinable whether unacceptable damaged is occasioned to surrounding tissue. In that regard with trials conducted to date, whilst some minor scarring has occurred the level of damage to the skin has been minimal. The treatment has led only to a transient eschar, with no particular evidence of discomfort to lambs.

To date the means by which the collagen-cleaving enzyme has been introduced is via injection by syringe. The needle in the above case is inserted into the dermis delivering a volume of about 0.1ml. It will be understood that the volume delivered and the method of delivery can be varied. The introduction of a volume of enzyme solution as effected in present trials, forces the collagen cleavage enzyme to spread throughout the dermis, and thus the pressure of the volume that is introduced should assist with spreading the enzyme and therefore the depilatory effect. Where only a minimal volume of some microlitres is delivered without the application of pressure the interstitial fluids within the dermis might be expected to carry the enzyme from the site of introduction but perhaps not distributing the enzyme to the same degree.

The site of delivery is preferably the dermis however it is anticipated that a subcutaneous delivery may well also be effective, providing enough contact with collagen network associated with the hair follicle of the area desired to be treated. It will be understood that the method of delivery may be by breaching the skin (for example, by injection or high pressure aerosol) or by application of a cream or other dermatological carrier with properties allowing delivery of the enzyme through the protective layers of the skin.

It will be understood that this method is ideally suited as a means of depilation in the breech of a sheep as a replacement of the present practice of mulesing sheep. It is anticipated that this method will have permanent effects over the life of a sheep, however, the method will still be useful should the process be required to be repeated periodically.

For a better understanding the invention will be described with reference to a number of examples.

BRIEF DESCRIPTION OF THE DRAWINGS.

Figure 1 shows the inhibitory effect of collagenase on fibre growth *in vitro*. Concentrations of collagenase greater than 0.001% inhibited fibre growth *in vitro*.

Figure 2 is a schematic drawing of a hair and its follicle showing the connective tissue (1); the hair fibre (2); the inner root sheath (3); the outer root sheath (4); the bulb (5) and the dermal papilla (6),

Figure 3 shows photomicrographs of (a) follicles plucked from a collagenase treated skin site showing the fibre (2), the bulb (5) and the inner (3) and outer (4) root sheaths; and (b) fibres plucked from a control site not treated with collagenase,

Figure 4 shows photomicrographs of skin sections (a) from a control site not treated with collagenase showing the follicle canal (7) from which the fibre (2) has been removed but the bulb and dermal papilla remain halfway (8) up the follicle canal towards the skin surface (9); and (b) from a collagenase treated site showing empty follicle canals (7),

Figure 5 is a histogram setting out the effectiveness of five different enzymes on depilation. The scale on the Y axis refers to the proportion of intact follicle bulb ends after plucking of skin treated with different enzymes (relative to the control = 1). For example a value of 3 means there were 3 times more

intact bulb ends following collagenase treatment than were apparent for the control skin

Figure 6 a) is a photographic view of a healed breech of an adult sheep that has been subjected to the mulesing procedure, b) is a photographic view of the breech of a lamb 6 weeks after having been subjected to collagenase treatment at two sites.

DETAILED DESCRIPTION OF THE INVENTION.

EXAMPLE 1 - Cultured follicle growth is inhibited by collagenase.

A thin strip of skin was removed from an anaesthetised area of the midside of a sheep. The skin strip was immediately placed in culture media (Williams E- Sigma Chemical Co.) and taken to the laboratory. Follicles were microdissected from the skin and placed in individual wells in a 24-well plate. Williams E Media was added to each well. Varying concentrations of collagenase were added to each well (0.1%, 0.01%, 0.001% and 0.0001% and 0% collagenase (Sigma crude collagenase Type 1A Sigma product no. C9891). Fibre growth was measured daily for 6 days. Detailed description of the in vitro culture method is presented in (Bates, Hynd, Penno and Nancarrow 1997- British J. Dermatology 137: 498-505. See figure 1 for results.

EXAMPLE 2 Treatment of pig skin

Methods

A dead piglet was obtained and the skin surface was cleaned using 70% ethanol. A collagenase mixture (1% w/w of Type IV, Type II, Type XI and Type V collagenases (Sigma Pharmaceutical, Australia)) in soft, white paraffin (Prosana Laboratories, Queensland, Australia) was painted onto one midside of the dead pig and the painted area of skin was heated using an infrared lamp at a distance of about 25 cm from the skin surface for 30mins. After this time the pig was rotated and the opposing midside was painted with soft, white paraffin containing no collagenase. This skin surface was also heated for 30mins using the infrared lamp as before. After cooling for approximately 10mins the paraffin and paraffin/collagenase mixtures were scraped from the skin surface.

Fibres were then plucked from each side using forceps and mounted onto microscope slides with DePeX for microscopy. Both treated areas of skin were then waxed using a commercially available home wax treatment (Nair Easiwax® strips). Biopsies of skin were collected from these hairless skin sites and fixed, processed and sectioned for histology. The skin sections were stained with SACPIC staining.

Results

Gross Observations

Fibres plucked from the collagenase/paraffin treated site came away from the skin very easily compared with the paraffin treated site where more force had to be applied and fibre tearing could be felt.

Microscopy of Fibres

Fibres plucked from paraffin treated control site:

No fibres were observed with any outer root sheath (ORS) attached to them. Some of the fibres had rounded, hollow ends with a gap left where the dermal papilla and remaining bulb cells would normally sit. The remaining fibres had torn, brush like ends where the fibre had broken at the keratogenous zone. The occasional fibre had wavy, stringy fragments of tissue attached to the fibre shaft that are thought to be sections of inner root sheath (IRS) and a pointy fragment of tissue at the bulb end that could be dermal papilla but there was still no ORS present.

Fibres plucked from collagenase treated site:

100% of the fibres plucked out and mounted had been removed from the skin in their entirety. All of the fibres clearly had a visible dermal papilla and an entire ORS encapsulating the follicle. Some of the follicles had been pulled out in clumps and epidermal tissue was still attached to them.

Microscopy of Skin Sections

Skin from paraffin treated site:

Some fibres that have clearly been pulled on by the waxing treatment show that the fibre has been removed but that the bulb end and dermal papilla have been left halfway up the follicle canal towards the skin surface. The fibre has broken at the keratogenous zone. Other follicle canals appear to be empty except for a group of cells at the very bottom that are highly vacuolated in appearance and have not yet been identified. In these cases the ORS was missing as well. The rest of the follicles have remained completely intact in the skin

with the fibre broken off at the epidermis and have only a slightly altered appearance due to the pulling force applied to them during waxing.

Skin from collagenase/paraffin treated site:

Empty follicle canals were seen in the tissue with no remaining intact follicles present. These canals also have large vacuolated cells at the bottom of the canals. The dermal sheath appears to be still present in the tissue but all traces of the follicle (fibre, IRS, ORS dermal papilla, bulb) are missing.

Conclusion

The control tissue treated only with paraffin and heat still has evidence of cells of the dermal papilla and ORS and bulb left *in situ* and these cells have the potential to form a new hair fibre. All of the above results and observations suggest that the entire follicles have been removed from the skin when it has been subjected to collagenase/paraffin and heat. There are no traces of the cells thought to have the potential to produce a new follicle and hair fibre.

EXAMPLE 3. Testing of different enzymes

The crutch of live sheep were tested for the effectiveness of a range of enzymes to assess the likely range of enzymes that might have an effect.

The following enzyme solutions were prepared: 0.1% collagenase (crude collagenase Type 1A Sigma Product no. C9891 made in saline with $CaCl_2$ 0.13g/l, collagenase sigma blend Type F (Sigma Product no. C7926) made in saline with $CaCl_2$ 0.13g/l, 0.1% dispase I (Roche Diagnostics Australia Product no. 210455 made in saline with no $CaCl_2$, 0.5% trypsin/EDTA Gibco BRL Product no. 15405-012 made in saline with no $CaCl_2$, 0.1% MMP3 (stromelysin, transin, proteoglycanase) Sigma Product no. M1677 made in saline with $CaCl_2$ 0.13g/l. For each enzyme 1 ml was injected dermally. Approximately 2 hours later, the wool fibres on injected sites were plucked manually using forceps. Plucked fibres were placed on microscope slides with paraffin oil and the number of intact follicles versus broken follicle ends counted.

A repeat of this plucking experiment was done *in vitro* to allow greater control over enzyme application. The results confirmed those obtained *in vivo*, that is, collagenase allowed

entire, intact follicles to be plucked from the skin after collagenase treatment. Trypsin and dispase were ineffective relative to collagenase while MMP3 was intermediate in effect (Figure 5).

We conclude that the most effective enzyme group for depilation is the matrix metalloproteinases and most particularly the collagenases.

In vitro

A range of concentration of collagenases (0%, 0.1%, 0.01%, 0.001% and 0.0001%) were used as for in vivo testing of hair fibre growth in harvested hair follicles. Concentrations of collagenase greater than 0.001% inhibited fibre growth in cultured follicles.

EXAMPLE 4 Alternative to mulesing of sheep - first trial

A trial using 12 lambs was conducted to determine the effectiveness of treating the breech of lambs to remove wool as an alternative to the presently widespread practice of mulesing. 0.1ml of a 0.1% aqueous preparation of collagenase was injected about 1mm beneath the surface of the skin at 6 different sites around the breech.

It was found that simple hand application worked effectively causing minimal discomfort to the lambs.

Six weeks later the injected sites were examined and found to contain evidence of thin scar formation followed by sloughing of the scab rendering the area of skin hairless. The hairless skin appears to be healthy, pink and soft with a slight scar ridge under the surface.

Figure 6a shows a healed mulesed area on an adult sheep. Figure 6b shows collagenase treated areas on the breech of a lamb 6 weeks following treatment.

On inspection about 4 months after injection the site remained hairless.

EXAMPLE 5 Alternative to mulesing of sheep second trial

8 sheep were treated with a solution of collagenase (0.5% collagenase (Sigma Product no. C7926) in phosphate buffered saline with Calcium (0.13g $CaCl_2$ /l). 0.1ml of collagenase

solution was injected into 4 sites on either side of the tail and 2 sites on top of the tail. It is anticipated that perhaps less injection sites may be required.

Inspection of these sheep at weaning (12 weeks of age and approx. 6 weeks after treatment) revealed no detrimental effects of the treatment.

It will be understood that this invention is applicable to a range of procedures for hair removal in animals and is not limited to solely being an alternative to mulesing. It might be used for example as an alternative to pizzledropping in male sheep. Removal of hair from animals other than sheep is also contemplated by this invention. Examples include, tattooing of skin in dogs, horses, cats and other domestic animals, and removal of hair from pigskin prior to slaughter.

Dated this 9th day of January 2002

ADELAIDE UNIVERSITY
By their Patent Attorneys
A.P.T. Patent and Trade Mark Attorneys

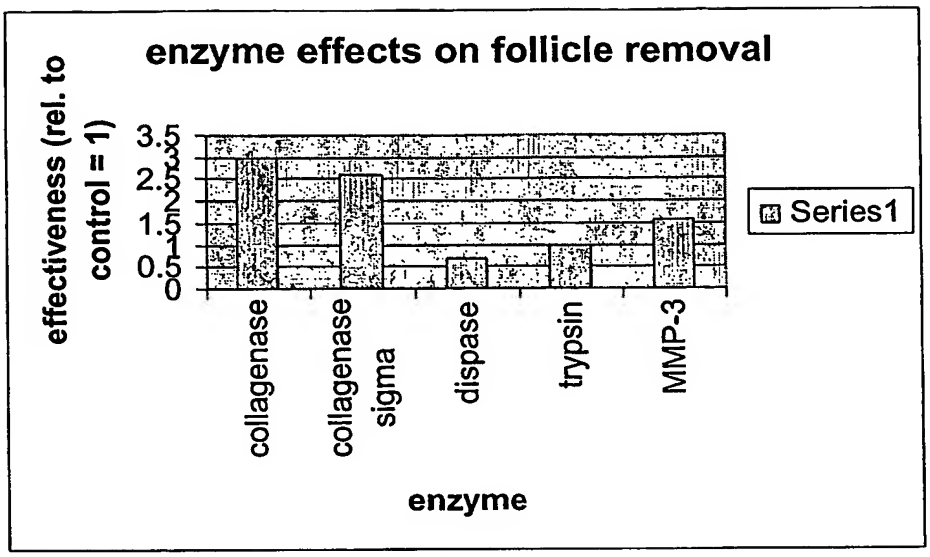

Figure 5

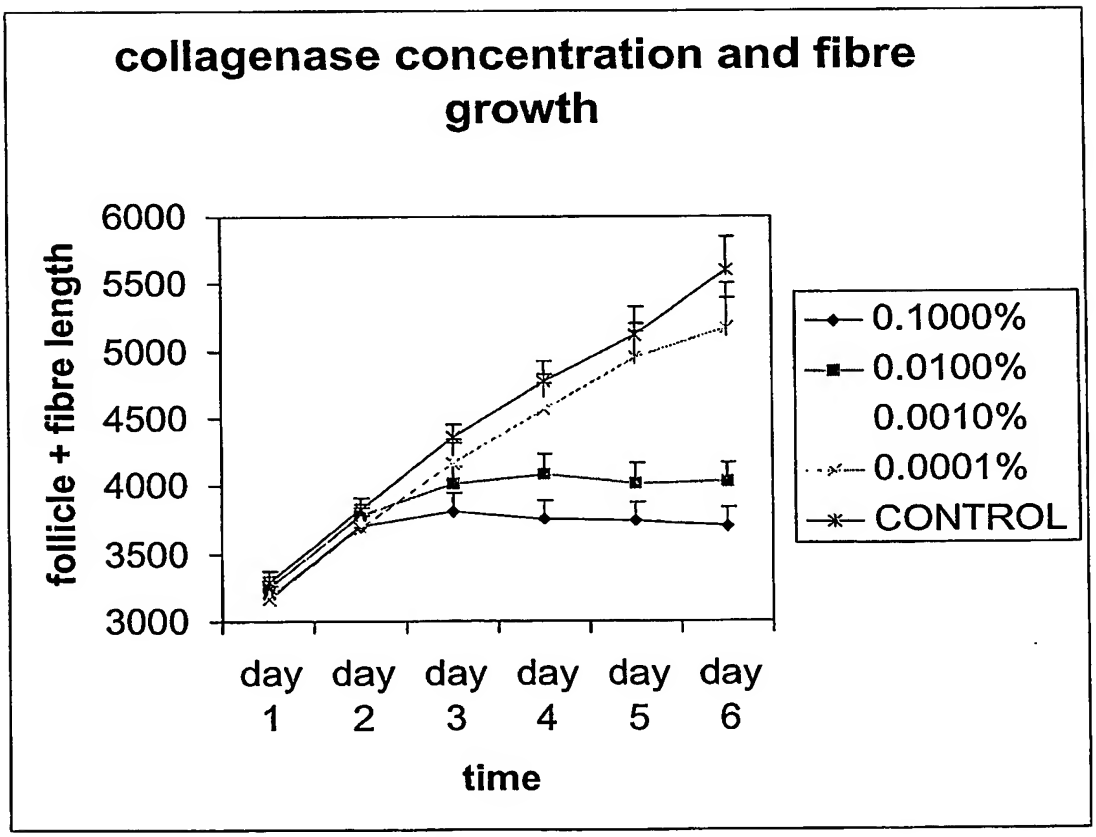

Figure 1

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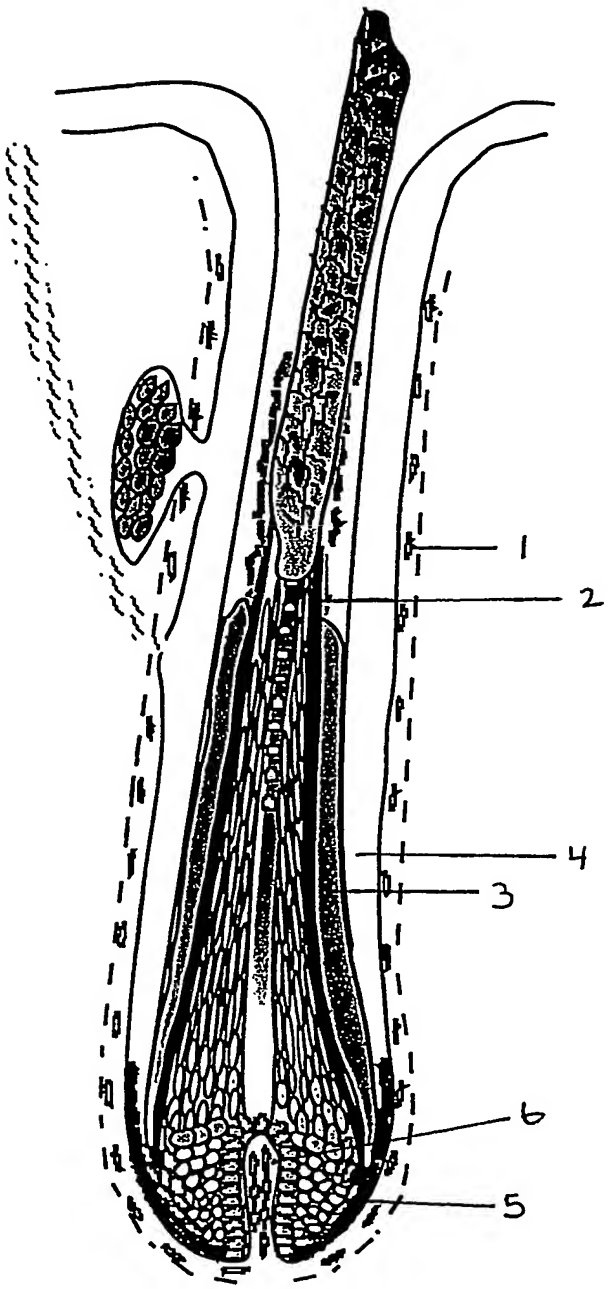

FIGURE 2

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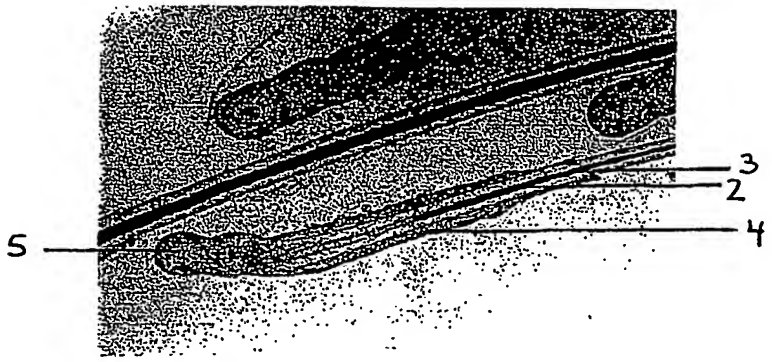

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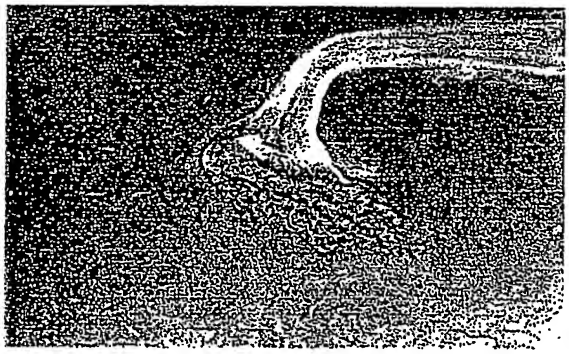

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FIGURE 3

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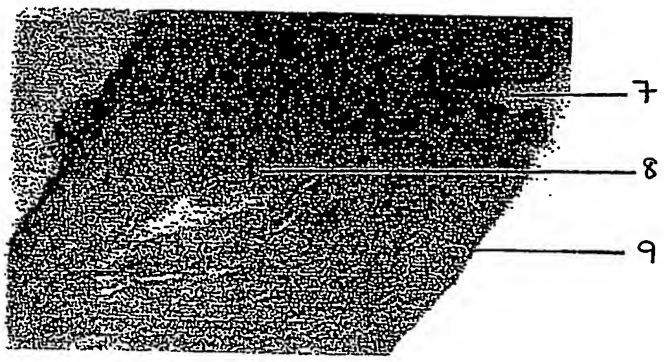

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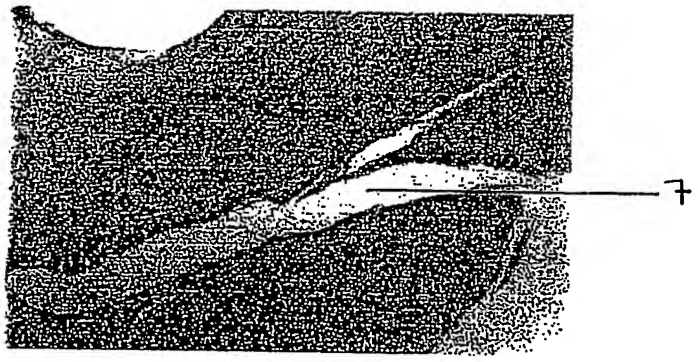

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FIGURE 4

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Figure 6a

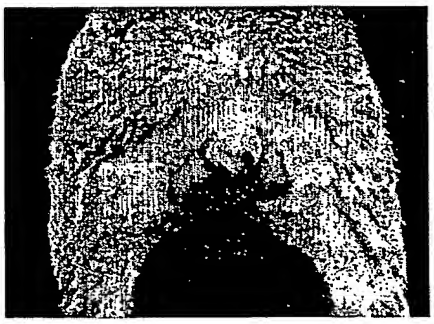
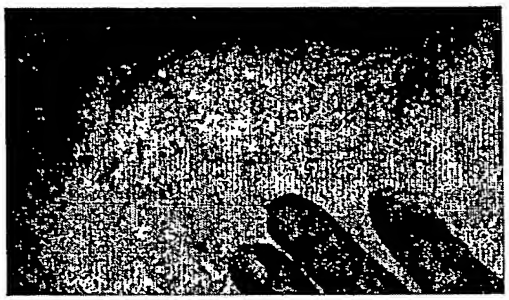


Figure 6b



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